Vitamin A deficiency impairs contextual fear memory in rats: abnormalities in glucocorticoid pathway

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Short title : Vitamin A, glucocorticoids and memory

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Abstract

Vitamin A and its active metabolite, retinoic acid (RA), play a key role in the maintenance of cognitive functions in the adult brain. Our previous work has shown that depletion of RA using the vitamin A deficiency (VAD) model in Wistar rats leads to spatial memory deficits in relation to elevated intrahippocampal basal corticosterone (CORT) levels and increased hippocampal 11β-Hydroxysteroid Dehydrogenase type 1 (11β-HSD1) activity. All these effects have been normalized by vitamin A supplementation. However, it is unknown whether vitamin A status also modulates contextual fear conditioning (CFC) in a glucocorticoid-associated fear memory task dependent on the functional integrity of the hippocampus. Here, we investigated the impact of VAD and vitamin A supplementation in adult male rats on fear memory processing, plasma CORT levels, hippocampal retinoid receptors and 11β-HSD1 expression following a novelty-induced stress. We also examined whether vitamin A supplementation or a single injection of UE2316, a selective 11β-HSD1 inhibitor, known to modulate local glucocorticoid levels, had any beneficial effects on contextual fear memory and biochemical parameters in VAD rats. We provide evidence that VAD exhibits a decreased fear conditioning response during training with a poor contextual fear memory 24 h later. These VAD-induced cognitive impairments are associated with elevated plasma CORT levels in basal conditions, and following a stressful event, with a saturated CORT release, altered hippocampal retinoid receptors and 11β-HSD1 expression. Vitamin A supplementation normalizes VAD-induced fear conditioning training deficits and all biochemical effects, but cannot prevent fear memory deficits. Moreover, a single injection of UE2316 impairs contextual fear memory but also reduces plasma CORT levels regardless of the vitamin A status and decreases slightly hippocampal 11β-HSD1 activity in VAD rats following stress. This study highlights the importance of vitamin A status in modulating fear memory conditioning in relation to plasma CORT levels and hippocampal 11β-HSD1.

Keywords: Vitamin A, retinoic acid receptor, glucocorticoid, 11β-Hydrosteroid Dehydrogenase type 1, contextual fear memory, hippocampus.

1. Introduction

Vitamin A and its active metabolite, retinoic acid (RA), play a key role in the maintenance of diverse functions in the adult brain by regulating neuroplasticity in cerebral structures including the hippocampus (1). Deleterious effects on hippocampal functions have been evidenced by using the vitamin A deficiency (VAD) model in rodents (2-3) a nutritional approach which leads to impairment in RA signaling (4-5). VAD disrupts hippocampal plasticity (6-9) and leads to cognitive deficits in hippocampus-dependent spatial memory tasks (2-3, 10) possibly by modulating gene transcription through its specific nuclear receptors (RARs: retinoic acid receptor and RXRs: retinoid X receptors) (5, 11). Interestingly, vitamin A supplementation or RA treatment can suppress hippocampal plasticity alterations and spatial memory deficits in VAD and in aged rodents, both of which exhibit decreased RA signalling (3, 12-14). Taken together, these data demonstrate a central role of the RA signaling pathway on hippocampus-dependent spatial memory and potential neurobiological mechanisms by which vitamin A status could modulate these memory processes.

More recently, the potential role of glucocorticoid signalling on VAD-induced cognitive impairments has emerged (15). It is well-established that glucocorticoid (GC; corticosterone in rodents and cortisol in humans) hormones, induce inverted U-shaped dose effects on different memory processes (16-17). Moreover, the magnitude of hippoccampal GC action on hippocampus-dependent memory processes is thought to be determined by free GC hormones circulating in blood which are delivered to the brain but also by local hippocampal activity of 11β-Hydroxysteroid Dehydrogenase type 1 (11β-HSD1). This enzyme regenerates active GC from its inactive forms (18), thereby effectively amplifying intracellular GC signalling (19). It has been shown that short term treatment of the selective 11β-HSD1 inhibitor, UE2316, which lowers intrahippocampal GC levels, reverses spatial memory impairments with ageing while reducing the strength and persistence of new contextual fear memories (20).

We have recently demonstrated that excess basal intrahippocampal corticosterone (CORT) levels probably resulting from both elevated free basal plasma CORT levels and hyperactivity of hippocampal 11β-HSD1 could contribute to spatial memory deficits in VAD rats (15). Interestingly, vitamin A supplementation can suppress spatial memory deficits and hippocampal neurogenesis alterations in VAD rats possibly by decreasing basal intrahippocampal CORT levels. In line with these results, we have also demonstrated that the

stimulation of the retinoid signaling pathway could be a viable strategy to reverse cognitive alterations in middle-aged mice by reducing excess levels of intrahippocampal CORT collected by microdialysis following a novelty-induced stress (21). These studies suggested that the modulation of circulating and hippocampal GC levels by vitamin A status could be important neurobiological mechanisms by which this vitamin influences spatial memory processes. While vitamin A supplementation is beneficial in normalizing VAD- related spatial memory deficits, it is unknown if vitamin A status could also modulate contextual fear conditioning which is dependent on functional integrity of the hippocampus (22). Modulation of vitamin A status may thus provide an avenue to examine the extent to which VAD could lead to hippocampal dysfunctions.

In this study, we examined the impact of VAD on contextual fear conditioning, plasma CORT levels, hippocampal retinoid receptors and 11β-HSD1 mRNA expression, following a novelty-induced stress (Experiment 1). Moreover, as elevated hippocampal 11β-HSD1 activity is associated with spatial memory deficits in VAD rats, we also examined whether vitamin A supplementation or a single injection of UE2316, a selective 11β-HSD1 inhibitor known to modulate local GC levels, has beneficial effects on contextual fear memory and biochemical parameters following stress exposure in VAD rats (Experiment 1 and 2).

2. Materials and methods

2.1 Animals

Weaned male Wistar rats (3 weeks old) were purchased from Janvier (Le Genest Saint-Isle, France). They were housed two per cage in a room with a constant airflow system, controlled temperature (21-23°C), and a 12h light/dark cycle. Rats were given *ad libitum* access to food and water and weighed twice a week. As in (3), one week prior to the beginning of behavioral experiments, all animals were housed two per cage until sacrifice. All experiments were performed in accordance with the European Communities Council Directives (2010/63/EU) and the French application texts of this directive, and have been approved by the Animal Care and Use Committee of Bordeaux under the N°50120169-A.

2.2 Experimental design

2.2.1 Experiment 1: effects of vitamin A status on contextual fear conditioning

On arrival, weaned rats were randomly assigned to two experimental groups: one group (n=20) was fed with a control diet containing 5 IU retinol/g (INRA, Jouy-en-Josas), whereas the second one (n=20) received a vitamin A-free diet (0 IU retinol/g) (Laboratorio Piccionni, Italy) for 10 weeks. They are referred to as control rats (n=20) and vitamin A deficient rats (VAD) (n=20), respectively. In the first experiment, we studied the effects of vitamin A status (deficiency and supplementation) on contextual fear conditioning (**Fig. 1**). After 10 weeks of diet, the two experimental groups (Control n=20, VAD n=20) were tested in the actimetry test with a systematic characterization of locomotor activity. Then, half of the vitamin A deficient rats (n=10) and half of the control rats (n=10) were supplemented with a vitamin A-enriched diet (20 IU retinol/g) during the next 4 weeks until sacrifice: these groups were referred to as VAD + Vit A and Control + Vit A, respectively while the other half were kept on their respective diets.The supplemented vitamin A diet (20 IU retinol/g) was used, as it has been shown to be effective in reversing VAD-related memory decline (15). Thus, 12 weeks after their arrival, rats were trained and tested in a contextual fear conditioning task. One week after fear conditioning, an open field test was performed to test locomotor activity. One day before this test, blood samples collection from tail vein was performed for basal plasma CORT measures. Then, all groups were euthanised 20 min after this mild stressor

exposure to collect blood samples and hippocampi for further post-stress biochemical and molecular analyses.

2.2.2 Experiment 2: effects of UE2316 treatment on contextual fear conditioning

In the experiment 2, we studied the involvement of 11β-HSD1 on the effects of VAD on contextual fear conditioning. The novel compound UE2316, the 11β-Hydroxysteroid Dehydrogenase type 1 inhibitor provided by SP Webster, has been shown to be a potent enzyme inhibitor in control mice with a single intraperitoneal injection at 10 mg/kg and to reduce plasma CORT levels after a novel environment induced-stress (23). We firstly verify the capacity of the inhibitor, injected intraperitoneally one hour before the training in the fear conditioning chamber, to reduce plasma CORT levels in control rats. Thus, a first group of weaned control rats received the control diet (5 IU retinol/g; n=10) during 12 weeks and were divided into two subgroups: the rats received a single injection of the vehicle (Control + Vehicle n=10), or the 11β-HSD1 inhibitor UE2316 (10 mg/kg) (Control + UE2316, n=10). Then, control rats were euthanised 20 min after the fear conditioning induced-stress to collect blood samples for further measurement of CORT levels.

A second group of weaned rats received a vitamin A-free diet (0 IU retinol/g; n= 20) or the control diet (5 IU retinol/g; n=20) during 12 weeks and were divided into two subgroups: the rats received a single injection of the vehicle (Control + Vehicle n=10;VAD + vehicle, n=10), or the 11β-HSD1 inhibitor UE2316 (10 mg/kg) (Control + UE2316, n=10; VAD+ UE2316, n=10). The inhibitor was dissolved in a mixture (vehicle) containing DMSO-NaCl-polyethylene glycol (2:38:60, by vol.) and injected intraperitoneally one hour before the training in the fear conditioning chamber but also one hour before the open field test performed in the same conditions than those of the experiment 1. Then, all groups were euthanised 20 min after this mild stressor exposure to collect blood samples and hippocampi for further post-stress biochemical analyses.

2.3 Behavioral tests

2.3.1 Actimetry test

10 weeks after their arrival, the spontaneous locomotor activity of the rats was recorded for 30 min in actimetry cages (20 X 33 X 18 cm, Imetronic, Pessac, France) before

vitamin A supplementation and UE2316 treatment. Two infrared light beams passing through each cage were targeted on two photocells, 4 cm above floor level and 20 cm apart. The number of cage crossings by the rats was recorded by a computer and accounted for the horizontal activity. Blocks of 5 min were considered for statistical analysis.

2.3.2 Contextual Fear Conditioning

After 12 week-VAD (and 2 week-vitamin A supplementation for the first experiment), all groups of rats were conditioned in eight identical chambers (40 cm x 35 cm x 30 cm; Imetronic, Pessac, France) made of gray polyvinyl chloride on three sides and transparent Perspex on the door. Each chamber was located inside a sound-attenuating cubicle with a roof-mounted loudspeaker and a ventilation fan providing background noise of 55 db. Behaviour was recorded from above through a wide-angle (2.5) mini camera (SK-2005, Opto Vision, Toulouse, France). The grid floor (27 parallel 0.5-cm-diameter stainless-steel bars, 1.5 cm apart placed above a sawdust tray) delivered mild foot shocks (US, 1s, 0.4-mA scrambled pulses). The computerized (Imetronic) conditioning procedure consisted of unpaired delivery of five tones (5,000 Hz, 70 dB, 10 s in duration) and five electric shocks (the contingency was irregular) over an 8-min period (no stimuli for the first 2 min). This procedure promotes conditioning to the context (24-25). The context test took place 24 h later by placing rats in the same conditioning chamber for 5 min without any stimuli. For the tone test performed 24 h after the context test, the conditioning chambers were modified to define an altered context and to minimize residual contextual fear. Furthermore, each animal was tested in another chamber than the one used for conditioning. Black and white visual patterns were fitted on the front and transparent wall to alter the visual features of the environment. Tactile cues were altered by placing a granulated plastic plate with a checkerboard visual pattern on the grid floor. For automatic freezing analysis, data acquisition was carried out by a program written under the TestPoint software, according to a previously validated method (24-25). Briefly, images of the conditioning chamber, sampled at 1 Hz, were subtracted from a previously recorded image of the empty cage and the contrast of the resulting image (standard deviation of pixel values) was computed. A change score was then calculated as the standard deviation of contrast over three successive frames. The rat was considered to freeze when the change score was lower than a fixed threshold (0.075) over two successive frames. Freezing levels correspond to the percentage of time spent freezing over a specified duration (ranging from 1 to 5 min as indicated).

2.3.3 Open-field test

This test measures the reactivity to novelty of the animals on placement in a novel environment after 13 weeks of VAD. Each rat was individually placed for a 10-min session in a circular open field filled with bedding (1m diameter, 50 lux at the center). A low light intensity and bedding were used to limit high anxiety levels and to obtain normal amount of exploration levels. The 30-cm high wall was made of clear Perspex, and numerous distal and proximal cues were provided to encourage rats to explore this environment. A videotracking system (Viewpoint, Lyon, France) recorded the path length travelled by rats, with activity analyzed using 2-min blocks.

2.4 Tissue preparation

Blood samples were also collected from tail vein in order to measure basal plasma corticosterone levels (CORT) one day before the openfield test. 20 min after the openfield test, rats were transferred to a room adjacent to the laboratory, were euthanised with isoflurane and decapitated within 3 min to avoid the effects of euthanasia on plasma (CORT) levels. As described in figure 1 (experiment 1 and 2), trunk blood was collected immediately in order to measure plasma CORT following stress. Trunk blood was then centrifuged to obtain plasma samples (1500 g for 10 min in tubes containing 10 % EDTA). The supernatant was collected and stored until assay at -20°C. In order to measure mRNA expression by quantitative RT-qPCR and 11β-HSD1 enzyme activity**,** hippocampi were rapidly removed, frozen in liquid nitrogen and then stored at -80°C until assay.

2.5 Plasma corticosterone (CORT) assay

The levels of plasma CORT were evaluated in basal conditions from tail vein before the open field test and post-mortem, 20 min after this mild stressor exposure. The DetectX corticosterone immunoassay kit (Arbor Assays) was used to quantitatively measure total corticosterone present in plasma. A corticosterone stock solution was used to generate a standard curve (from 10000 to 78.125 pg/ml) for the assay and all samples were quantified from the standard curve. According to the manufacturer’s protocol, the concentration of the

corticosterone in the sample was calculated, after making a suitable correction for the dilution of the sample, using a microplate reader (Victor 3V, PerkinElmer).

2.6 Index of adrenal gland

The adrenal glands were removed and weighed immediately post-mortem. The index of adrenal gland was expressed as the ratio of adrenal gland weight to 100 g of body weight.

2.7 Real-Time PCR analyzes of retinoid target gene expression in the hippocampus

For the first experiment, hippocampi were used to measure gene expression. RNA extraction was conducted using Trizol reagent (Invitrogen, Saint Aubin, France) according to the manufacturer's instructions. The integrity of the purified RNA was verified using the RNA 6000 Nano LabChip kit in combination with the 2100 Bioanalyzer (Agilent Technologies). All RNA samples scored a RIN (RNA Integrity Number) ≥ 8, suggesting high quality RNA sampleswithout fragmentation.The concentrations of RNA and absence of chemical contamination (for example Trizol) were determined by using a Nanodrop ND-1000 (Ozyme). Using oligodT and random primers (Promega, Charbonnières les bains, France), cDNA was synthesized from 1µg of RNA with ImPromII reverse transcriptase (Promega, Charbonnières les Bains, France) according to the manufacturer protocol. The real-time PCR was performed using the LightCycler 480 system with a 96-well format (Roche Diagnostics) in a volume of 20µL, containing 1X Light Cycler 480 SYBR Green I Master solution, 0.5µM of each primer and 6µL of cDNA. In this study, we used the BMG housekeeping gene as the reference mRNA since its expression level was unaffected by our experimental conditions. The stability of BMG mRNA was checked by comparing its Cp values in the different experimental groups after a RT-qPCR. The analysis showed no statistically significant variation of the BMG mRNA among the four different experimental groups [$F(3,37) = 0.789$, n.s]. The forward and reverse primer sequences for the 11β-HSD1, RARα, RARβ, and β2-microglobulin (BMG) are given in Table 1.

2.8 11β-HSD1 enzyme activity in the hippocampus

For the second experiment, hippocampi were homogenized on ice in 1mL of buffer (1.37M Glycerol, 300mM NaCl, 1mM EDTA, 50mM Tris, 1X Phosphatase Inhibitor

Cocktail, 2mM NaOV, 1mM NaF; pH=7.7). The total protein content of the homogenate was determined with ABC Assay kit (Uptima, Montluçon, France). The measure of 11β-HSD1 activity from hippocampus homogenates has been previously described by Bonhomme et al. (15). In vivo, 11β-HSD1 catalyzed the conversion of inactive 11-dehydroCORT to CORT. According to Moisan et al. (26), dehydrogenase activity was measured by quantifying the conversion of CORT (B) to 11-dehydroCORT (A). 0.5 mg/mL of total protein were incubated at 37°C for 1h with 12nM ^{3}H-CORT as substrate (specific activity: 78.1Ci/mmol, PerkinElmer) and an excess (400µM) of the enzyme-specific cofactor NADP. After incubation, steroids were extracted by addition of ethyl acetate, separated by thin-layer chromatography on silica gel plates (TLC Silica Gel 60 F254, VWR) using a mixture of chloroform and ethanol (92:8). Then, ^{3}H-CORT and ^{3}H-dehydroCORT were quantified with a β-Imager apparatus and 11β-HSD1 activity was expressed as the percentage conversion of ^{3}H-CORT (B) to ^{3}H-dehydroCORT (A).

2.9 Statistical analysis

Spontaneous locomotor activity in the actimetry test was analysed by a 1-way ANOVA with repeated measures (effect of deficiency and time). Contextual fear memory, tone test, path lengh in the open field test, plasma CORT levels, PCR data and 11β-HSD1 enzyme activity were analysed using a 2-way ANOVA (effect of deficiency and supplementation for experiment 1 and effect of deficiency and UE2316 treatment for experiment 2) followed by a *post-hoc* Fisher PLSD test. Training data were analysed using a 3-way ANOVA with repeated measures (effect of deficiency, supplementation and time for experiment 1; effect of deficiency, UE2316 treatment and time for experiment 2) followed by a *post-hoc* Fisher PLSD test. Kinetic analysis of plasma CORT release (Basal vs Stress) were performed using a 3-way ANOVA with repeated measures (effect of deficiency, supplementation and stress conditions) followed by a *post-hoc* Fisher PLSD test. All results were expressed as mean ± SEM.

3. Results

3.1 Experiment 1: effects of vitamin A status on contextual fear conditioning in relation to plasma CORT levels and hippocampal 11β-HSD1 following stress

3.1.1 Effects of 10 week-VAD on spontaneous locomotor activity in the actimetry test.

The impact of vitamin A deficiency on spontaneous locomotor activity was evaluated at 10-week VAD in the actimetry test (**Fig. 2**). A 2-way ANOVA on actimetry index over the 30 min revealed no significant differences across the groups [deficiency, $F < 1$; deficiency x time, $F < 1$] and activity declined across time in all groups [time, $F(5,195)=52.5$, $p<0.0001$]. Thus, the actimetry index indicated that a 10-weeks VAD does not induce alterations in global locomotor activity.

3.1.2 Effects of VAD and vitamin A supplementation on contextual fear conditioning

Conditioning

At 12-week VAD, animals were trained in a fear conditioning chamber. As shown in **Fig. 3A**, all groups showed acquisition of freezing behavior over the 8 minutes of the conditioning session [time, $F(7, 259)= 61.84$, $p<0.0001$]. Freezing behavior appeared however to be largely reduced in the VAD group that did not receive vitamin A supplementation. These observations were supported by highly significant effect of deficiency [$F(1,37)=11.48$, $p<0.0017$] and supplementation [$F(1,37)= 5.8$, $p< 0.0001$]. More precisely, a significant decreased freezing level was observed at 5, 7 and 8 min in VAD rats compared to controls [5 min : $F(1,19)=11.18$, $p=0,0034$; 7 min : $F(1,19)=7.82$, $p=0.01$; 8 min : $F(1,19)=9.63$, $p=0,005$]. Furthermore, while the deficiency x supplementation and the deficiency x supplementation x time interactions did not reach significance [$F<1$; $F(7,259)=1.7$, $p=0.1208$, respectively], highly significant deficiency x time [$F(7,259)=4.9$, $p<0.0001$] and supplementation x time [$F(7,259)=3.50$, $p=0.0014$] interactions were evident. We therefore took this opportunity to verify that freezing increased both in control and VAD groups [time, $F(6,108)=41.6$, $p< 0.0001$; time, $F(6,114)=15.0$, $p<0.0001$, respectively]. Finally, the critical time x supplementation was significant for the VAD [$F(6,114)=2.34$, $p=0.0366$] but not for

the control group [F(6,108)=1.6, p=0.1594], suggesting that the vitamin A supplementation restored normal freezing behavior in VAD rats during conditioning.

Contextual fear test

Twenty-four hours after conditioning, contextual fear memory was assessed by placing rats again in the same conditioning chamber. During this context test, freezing levels were relatively low, suggesting only moderate expression of conditioned fear to the context in all groups (**Fig. 3B**). However, lower levels of freezing were detected in VAD rats as shown by the significant effect of deficiency [F(1,37)=4.2, p=0.0475]. Even if levels of freezing appeared to be slightly higher in VAD rats that received supplementation, this observation was not confirmed by the ANOVA as neither the main effect of supplementation [F<1] nor the supplementation x deficiency interaction [F(1,37)=1.3, p=0.2603] reached significance.

Tone test

Twenty-four hours after the context test, a tone test was conducted to examine whether rats would express fear to the stimulus. This test was conducted in a new environment. The data clearly indicated a freezing behavior during tone presentation in all groups (**Fig. 3C**). Indeed, neither the main effect of deficiency, supplementation nor the deficiency x supplementation (F<1) reached significance during the tone test. Thus, normal levels of freezing during the tone test were observed in VAD rats, suggesting that the impairments evidenced during the context test may not directly result from sensory processes alterations during acquisition (i.e. they were able to exhibit normal freezing behavior). Interestingly, unlike the control group, levels of freezing in VAD rats were increased during the tone test compared to the context test [deficiency x context vs tone F(1,37)=4.86, p=0.033], suggesting that VAD rats expressed an increased emotional response to an elemental cue that did not explicitly predict shock delivery [Context vs tone freezing levels : VAD: 11.75 ± 1.65 % < VAD: 25.5 ± 3.41 % respectively; Control: 27.83 ± 7.53 % = Control : 19.9 ± 5.039 % respectively].

3.1.3 Effects of VAD and vitamin A supplementation on locomotor activity in the open field test

One week after fear conditioning, all rats were tested in the open field test (**Fig. 4A**). Locomotor activity appeared to decline across time in all groups [time, $F(4,148)=15.54$, $p<0.0001$], suggesting that all rats gradually habituated to the new environment. A 3-way ANOVA applied over the 10 min period (5 blocks of 2 min) revealed no main effect of deficiency and supplementation across the groups [deficiency, $F<1$; supplementation, $F(1,39)=2.42$, $p=0.128$] nor any interactions [deficiency x supplementation, $F<1$; deficiency x time, $F<1$; supplementation x time $F(4, 148)=1.05$, $p=0.38$; deficiency x supplementation x time, $F<1$]. Altogether these results thus indicate that 13 weeks of VAD and a 3-week vitamin A supplementation had no effect on reactivity to novelty in the open field test.

3.1.4 Effects of VAD and vitamin A supplementation on total plasma corticosterone (CORT) levels and adrenal gland index

We determined total plasma CORT levels evaluated during basal conditions (one day before the open field test) and 20 min after the open field test-induced stress in the same animals. ANOVA on plasma CORT levels in basal conditions (**Fig. 4B**) revealed a deficiency tendency [$F(1,33)=3,53$, $p=0.06$] without supplementation effect [$F(1,33)=2.92$, $p=0,09$] and a significant deficiency x supplementation interaction [$F(1,33)=12.32$, $p<0.01$]. VAD caused a significant elevation of basal plasma CORT levels (Fisher's *post-hoc* VAD vs Control, $p<0.01$; VAD: 61.05 ± 15.4 ng/ml > Control: 14.76±3,91 ng/ml). The supplementation normalized the level of plasma CORT in VAD rats (Fisher's *post-hoc* VAD + Vit A: 16,23 ± 4.79 ng/ml < VAD, $p<0.01$), but had no effect in control rats (Fisher's *post hoc* Control vs Control + Vit A : 30.23 ± 5,54 ng/ml, n.s.). ANOVA on plasma CORT levels following stress exposure revealed a deficiency effect [$F(1,33)=11,3$, $p<0,01$] without supplementation effect [$F=1.6$] and deficiency x supplementation interaction [$F<1$]. Indeed, VAD groups with or without vitamin A exhibited a lower increase in plasma CORT levels post-stress compared to control group.

We then examined the kinetics of total plasma CORT release by comparing the levels of CORT between basal and post-stress conditions in each group. All groups showed increased CORT levels after the open field test [basal/stress, $F(1,33)=130.27$, $p<0.0001$]. However, the levels of total plasma CORT appeared to evolve differently between groups as indicated by

highly significant deficiency x supplementation x basal/stress interactions [F(1,33)=6.4, p=0.01]. Indeed, the kinetic analysis comparing plasma CORT levels in resting and post-stress conditions indicated no differences between basal and stress conditions in the VAD group, exhibiting a saturated post-stress CORT release [VAD: 61.05±15.4 ng/ml (basal) = 99.96 ±13.69 ng/ml (stress); Fisher's *post-hoc* basal compared to stress, n.s]. By contrast, the other groups (Control, Control + Vit A and VAD + Vit A) exhibited differences in plasma CORT levels during basal and stress conditions (Fisher's *post-hoc* basal compared to stress in each groups, p<0.0001). Thus, vitamin A supplementation normalized the kinetic of plasma CORT release (VAD + Vit A: 16.23 ± 14.37 ng/ml (basal) < 93.72 ± 8.23 ng/ml (stress)) in VAD group.

The release of plasma CORT is stimulated from the adrenal cortex. ANOVA on adrenal gland index (ratio of adrenal gland weight to 100 g of body weight) **(Fig. 4C**) revealed a supplementation effect [F(1,37)=15.59, p<0001] and a significant deficiency x supplementation interaction [F(1,37)=13.16, p<0.001]. These results showed the hyperplasia of adrenal cortex that could result from higher CORT biosynthesis in VAD rats. Supplementation with vitamin A normalized the adrenal gland index in VAD rats (Fisher's *post-hoc* VAD vs Control, VAD: 0.011 ± 0.001 g/100g > Control: 0.007 ± 0.001 g/100g, p<0.001; VAD > VAD + Vit A=0.005± 0.001 g/100g, p<0.0001).

3.1.5 Effects of VAD and vitamin A supplementation on hippocampal expression of retinoid receptors and 11β-HSD1.

We have previously shown that RA treatment modulates expression of RA receptors, especially RARα and RARβ in middle-aged mice. This finding correlates with the amplitude of the intrahippocampal CORT levels following a novelty-induced stress (21). In the present study, we investigated the influence of vitamin A status on hippocampal mRNA expression of these two RA receptors and 11β-HSD1, following the open field induced-stress. For gene expression, results were expressed in arbitrary units (a.u).

As seen in **Fig. 5A**, ANOVA performed on hippocampal RARα expression showed a supplementation effect [F(1,37)=4.66, p<0.05], indicating that the elevated hippocampal RARα mRNA expression in VAD rats was decreased by vitamin A (Fisher's *post hoc* VAD : 1.22 ± 0.063 a.u > control + VitA : 1.043 ± 0.04 a.u, p<0.05 and VAD + Vit A : 1.032 ±

0.075 a.u. < VAD). However, the deficiency x supplementation interaction did not reach significance [F(1,37)=1.26, n.s].

As seen in **Fig. 5B**, opposing effects of deficiency and supplementation have been observed on RARβ mRNA expression in VAD rats. Indeed, The ANOVA shows a tendency for a supplementation effect [F(1,35)=3.46, p=0.07] and a deficiency x supplementation interaction [F(1,35)= 10.71, p<0.01]. Indeed, VAD induced a decreased RARβ mRNA expression (-30%) compared to controls (Fisher's *post hoc* VAD vs Control, p<0.05; VAD: 1.43 ± 0.079 a.u. < Control: 2.018 ± 0.15 a.u.). Interestingly, the vitamin A supplementation normalized RARβ mRNA expression in VAD rats (Fisher's *post-hoc* VAD + VitA : 2.32 ± 0.24 a.u. > VAD, p<0.001), but had no effect in control rats.

The ANOVA on hippocampal mRNA expression of 11β-HSD1 following stress (**Fig. 5C**) showed a deficiency effect indicating an increased level of 11β-HSD1 mRNA expression (+ 14.5 %) in VAD rats compared to controls [F(1,36)=4.69, p<0.05] and a supplementation effect [F(1,36)=9.013, p<0.01] but no deficiency x supplementation interaction reached significance [F(1,36)=2.15, p=0.15]. Indeed, VAD induced an increased 11β-HSD1 mRNA expression (+14,5%) compared to controls (Fisher's *post hoc* VAD vs Control, VAD: 1.264 ± 0.051 a.u. > Control: 1,104 ± 0.034 a.u, p<0.05). Interestingly, the vitamin A supplementation in VAD rats decreased the levels of 11β-HSD1 mRNA expression in the hippocampus (Fisher's *post hoc* VAD >VAD + Vit A : 1.067 ± 0.04 a.u., p<0.01) but had no effect in control rats (Fisher's *post hoc* Control vs Control + Vit A : 1.036 ± 0.046, n.s.).

Moreover, correlation analyses have been performed between hippocampal expression of RARs and 11β-HSD1 mRNA expression in order to study interactions between RA and GC signaling pathways. Correlation analyses between hippocampal expression of RARα and 11β-HSD1 in VAD and VAD + Vit A groups showed that elevated expression of RARα is associated with a higher 11β-HSD1 mRNA expression (Fig. 5D, r= 0. 461, p=0.03). However no correlation was detected between hippocampal expression of RARβ and 11β-HSD1.

3.2 Experiment 2: effects of a single injection of UE2316 on contextual fear conditioning memory, plasma CORT levels and hippocampal 11β-HSD1 activity in relation to vitamin A status

We firstly verified the capacity of the 11β-HSD1 inhibitor to reduce plasma CORT levels in control Wistar rats. Interestingly, we found that a single injection of UE2316 (10 mg/kg) one hour before training in the fear conditioning decreased plasma CORT levels following this stressful event in control rats [$F(1,14) = 4,82$, $p<0.05$; Control + UE2316 : 260, 5 ± 32.9 ng/ml < Control + Vehicle : 378.9 ± 42.6 ng/ml].

As VAD induces an elevated expression of hippocampal 11β-HSD1, we next examined whether a single injection of UE2316, could have any beneficial effects on contextual fear memory and plasma CORT levels following an open field induced-stress in relation to vitamin A status using the same protocol used in experiment 1 (see Figure 1).

3.2.1 Effects of UE2316 on contextual fear conditioning in relation to vitamin A status

Conditioning

At 12-week VAD, the selective inhibitor UE2316 was injected in rats one hour before the training in the fear conditioning chamber. As shown in **Fig. 6A**, all groups showed acquisition of freezing behavior over the 8 minutes of the conditioning session [time, $F(7, 245)= 31.25$, $p<0.0001$]. Consistent with the first experiment, only low levels of freezing were evident in VAD rats. However, drug treatment appeared to have no effect on that measure and could not restore freezing level during conditioning in VAD rats. These observations were supported by a highly significant effect of deficiency [$F(1,35)=6.33$, $p<0.001$], while neither the main effect of UE2316 ($F<1$), nor any interaction with this factor reached significance [Deficiency x UE2316, $F<1$; Deficiency x UE2316 x Time, $F<1$]. As the Deficiency x Time was significant [$F(7,245)=2.85$, $p<0.07$], we therefore took this opportunity to verify that freezing gradually increased for both the control and the VAD group [$F(7,112)=21.55$, $p< 0.0001$; $F(7,133)=9.84$, $p<0.0001$, respectively]. Altogether these results showed that UE2316 treatment had no effect on freezing behavior in control and VAD rats during conditioning.

Contextual fear test

A 2-way ANOVA of the freezing data over the 5 min in the conditioning chamber revealed a significant effect of deficiency [$F(1,35)=5.3$, $p<0.02$] as previously observed in the first experiment but also an inhibitor effect [$F(1,35)=4.71$, $p<0.03$] without any inhibitor x deficiency interactions ($F<1$) (**Fig. 6B**). UE2316 treatment in VAD and control rats prior to training significantly reduced the freezing response measured during the retention test, 24 h after treatment (40% reduction between VAD + Vehicle : 10.6 ± 1.33 % vs VAD + UE2316 : 6.4 ±1.19% ; 56.5 % reduction between Control + Vehicle = 25.65 ± 9.4 % vs Control + UE2316 = 11.16 ± 3.15 %). These results indicated that acute UE2316 treatment induced deleterious cognitive effects in the control and VAD groups.

Tone test

The data clearly indicated no differences between all groups during tone presentation **(Fig.6 C)**. Indeed, no effect of deficiency or inhibitor nor inhibitor x deficiency interactions were evidenced during the tone test [$F(1,35)=3.06$, $p=0.08$; $F<1$, $F(1,35)=1.66$, $p=0.2$, respectively]. Thus, as observed previously normal levels of freezing during the tone test were observed in VAD rats but also in UE2316 treated rats suggesting that the impairments evident during the context test may not directly result from their capacity to express freezing behavior.

3.2.2 Effects of UE2316 on locomotor activity in the open field test in relation to vitamin A status

One week after fear conditioning, UE2316 was injected in VAD and control rats, one hour before the open field test (**Fig. 7A**). Locomotor activity appeared to decline across time in all groups [time, $F(4,148)= 55.37$, $p <0.0001$], suggesting that all rats gradually habituated to the new environment. A 3-way ANOVA applied over the 10 min period (5 blocks of 2 min) revealed no main effect of deficiency and UE2316 treatment across the groups [deficiency, $F<1$; UE2316 $F<1$], nor any interactions [deficiency x UE2316, $F<1$; deficiency x time, $F<1$; UE2316 x time $F(4, 148)=1.08$, $p=0.36$; deficiency x UE2316 x time, $F(4, 148) =1.88$, $p=0.11$]. Altogether these results indicated that UE2316 had no effect on reactivity to novelty in the open field test in relation to vitamin A status.

3.2.3 Effects of UE2316 on total plama CORT levels in relation to vitamin A status

As seen in the Figure 7B, when UE2316 was injected one hour before the open field test, we found a reduced plasma CORT levels following stress, regardless of the vitamin A status [UE2316 effect : $F(1,36)=6.8$, $p<0.05$]. Indeed, the ANOVA indicated no interaction between UE2316 treatment and deficiency [$F<1$]. Moreover, as observed in the experiment 2, a main deficiency effect [$F(1,35) =5.49$, $p<0.05$] indicated that VAD rats regardless of the UE2316 treatment exhibited a lower increase in plasma CORT levels following stress compared to controls.

3.2.4 Effects of UE2316 on hippocampal 11β-HSD1 activity in relation to vitamin A status

The activity of 11β-HSD1 within the hippocampus expressed as a percentage conversion and measured following stress was affected by the UE2316 treatment (Fig. 7C) [$F(1,35)=4.95$, $p=0.032$], with a deficiency effect [$F(1,35)=4.56$, $p=0.039$] and a high trend deficiency x supplementation interaction [$F(1,35)=4.05$, $p=0.05$]. Thus, VAD rats treated with UE2316 exhibited a much more significant decrease in 11β-HSD1 activity compared to controls (Fisher's *post hoc* VAD + vehicle vs VAD + UE2316, $p<0.01$; VAD + UE2316 : 22.8 ± 1.07 % < VAD + vehicle : 27.1 ± 1.04 %). Indeed, this treatment did not have any effect on hippocampal 11β-HSD1 activity in control rats (Fisher's *post hoc* Control + Vehicle : 27,2 ±0.9 % vs Control + UE2316 : 27±0.8 % , n.s.).

4. Discussion

The main findings from this study are: (1) VAD impairs acquisition and fear memory in a contextual fear conditioning paradigm, (2) vitamin A supplementation restores performance during fear conditioning but cannot prevent contextual fear memory deficits, (3) VAD-induced fear memory deficits associate with elevated basal plasma CORT levels and a blunted CORT release, adrenal gland hypertrophy and elevated hippocampal 11β-HSD1 expression following a novelty-induced stress and all these effects are normalized by vitamin A supplementation, (4) an acute UE2316 treatment cannot restore performance during training but impairs contextual fear memory, reduces plasma CORT levels following a novelty-induced stress regardless of the vitamin A status, and slightly decreases the hippocampal 11 β-HSD1 activity in VAD rats. Together, these data indicate the importance of vitamin A status in modulating fear memory processing, that has never been described previously, in relation to plasma CORT levels and hippocampal 11β-HSD1 activity, known to contribute to intrahippocampal CORT levels.

It has been previously shown that VAD in rodents can impair hippocampus-dependent memory as spatial memory (2-3, 10, 15). The hippocampus plays an important role in spatial and relational memory processes but other studies suggest that this structure is also required in contextual fear conditioning (22). Here, VAD-induced cognitive impairments consisted of altered acquisition of the conditioned response with a poor retrieval of contextual fear memory performed 24 h after conditioning. The measure of freezing behavior as an index of fear memory can be readily disrupted by non-mnemonic factors such as locomotion or nociception. Given that VAD rats exhibited a decreased freezing response during the conditioning, we cannot exclude a reduced shock sensitivity in these rats. However, our data on freezing levels during the tone test, which is not predictive of the shock in this paradigm and performed 24 h after the retrievial, indicated similar freezing levels between controls and VAD rats. Moreover, we show that VAD rats froze more during the tone test compared to the context text with a high fear response to the cue. Therefore, as VAD rats were capable of showing high levels of freezing during the tone test, we can suggest that VAD impairs performances during conditioning rather than affects sensory processes. Another possibility was that contextual fear memory impairments in VAD rats could be due to an increased global locomotor activity. This parameter, assessed in the actimetry and the open field test, is

normal in VAD rats indicating that the acquisition impairment caused by VAD was unlikely due to non-specific effects on locomotion during fear memory test. Interestingly, the present study shows that vitamin A supplementation can normalize freezing behavior during the conditioning response but the impairing effects of VAD on contextual fear memory is not reversed by the supplemented diet. It has been shown previously by our team that 4 weeks of vitamin A supplementation can improve learning and spatial memory deficits in VAD rats (15). We can suggest that two week-supplementation in VAD rats is sufficient to normalize performances during fear conditioning, but is not enough to modulate consolidation processes that could underlie VAD-induced fear memory deficits. It could be interesting to test for longer vitamin A supplementation duration or to manipulate vitamin A signaling during consolidation processes to clarify this point.

The most general view is that stress or GC levels induce inverted U-shaped dose effects on cerebral plasticity and cognitive functions (16-17, 27-32). Thus, elevated plasma GC levels as a consequence of impaired hypothalamic-pituitary-adrenal (HPA) axis negative feedback have detrimental effects on hippocampus-dependent memory processes in aged rodents (33-35) and humans (36). Glucocorticoids enter the brain and bind to the mineralocorticoid receptor (MR) and glucocorticoid receptor (GR), that are abundantly expressed in the hippocampus and the amygdala, brain structures that are particularly sensitive to elevated GC levels and involved in the modulation of the HPA axis function (37-39). In the present study, we demonstrated that VAD in Wistar rats induces an elevated circulating basal CORT levels, a saturated plasma CORT release after a novelty-induced stress and an increase ratio of adrenal glands index. Moreover, vitamin A supplementation can correct altered circulating GC levels and adrenal hypertrophy. The increase in the ratio of adrenal gland index indicates an increase of CORT biosynthesis by adrenal glands. This adrenal hypertrophy is usually considered as a marker of the hyperactivity of the HPA axis (37, 40) and as a key feature of fear memory deficits after traumatic stress (41).

Although the elevated basal plasma CORT levels evidenced in our VAD rats has been previously shown, the mechanisms involved in modulating VAD-induced circulating GC levels remain poorly understood (15, 42). Some studies have demonstrated that retinoids play an important role in the control of plasma GC levels, by acting on neuroendocrine areas such as hypothalamus, pituitary and adrenal glands (43-44). Interestingly, administration of RA reduces and normalizes excess cortisol secretion in a good proportion of Cushing's syndrome patients exhibiting reduced hippocampal volume and impaired performance on hippocampal learning tasks (45-46). Thus, the effect of vitamin A supplementation in VAD-induced HPA

axis dysregulation would be the result of the interplay between retinoid action at these different neuroendocrine areas. Another possible mechanism involved in the VAD-induced HPA axis dysregulation could be an altered feedback resulting from GR activity dysfunction in brain structures such as hippocampus or amygdala, known to be involved in the control of HPA axis. Indeed, a decreased GR expression evidenced in VAD LOU/C rats, or modifications of its phosphorylation induced by RA treatment in hippocampal HT22 cell line, could contribute to a less efficient feedback by CORT on HPA axis tone leading to chronic elevation of circulating basal CORT levels (42, 47). Moreover, the most innovative feature of the present study is that we have found a blunted plasma CORT release following stress in VAD Wistar rats that is prevented by vitamin A. Evidence shows that corticosteroid binding globulin (CBG), that regulates plasma free CORT levels, is particularly important for an appropriate response to stress (48-50). More precisely, in a recent study these authors show that CBG deficiency leading to a blunted rise of GC after an acute stress (48), impairs contextual fear memory (51). We have shown previously that VAD rats exhibit CBG deficiency (15) and the present study supports the interpretation that CBG deficiency in VAD rats could contribute to altered plasma CORT release following stress.

Although elevated plasma GC levels can induce spatial memory deficits (52), they also strengthen emotional processing in tasks such as contextual fear conditioning (53-54). Several studies demonstrate that the strength of memory in the fear conditioning task is related to the plasma CORT levels (55-56). Indeed, freezing to context is enhanced during training and retrieval when shock intensity increases with plasma CORT levels (56). Moreover, when plasma CORT release is reduced by metyrapone treatment during fear conditioning training in chronically stressed rats, contextual fear conditioning is eliminated while tone conditioning is reduced (57). In the present study, we have hypothesized that a blunted plasma CORT release during fear conditioning training could be involved at least in part in VAD-induced fear performance deficits leading to altered fear memory processing. However, vitamin A supplementation that restores normal plasma CORT levels and fear conditioning performances cannot prevent contextual memory deficits in VAD rats suggesting that VAD-induced fear memory impairments cannot only result from performance deficits but also from other neurobiological mechanisms possibly involved in consolidation processes.

Interestingly, when circulating GC levels are elevated, the hippocampus via its interaction with the amygdala plays a dissociable role in processing the contextual and emotional properties of a fear memory (22, 58-60). Thus, rats given chronic CORT in drinking water, a manipulation that causes hippocampal dendritic alterations (61), exhibit

enhanced freezing to context in a contextual fear conditioning paradigm but not to tone, in a shock-paired tone conditioning paradigm, which critically depends on basolateral amygdala function (62). Moreover, amygdala inactivation during fear impairs contextual and tone conditionned fear memory regardless of chronic CORT treatment (62-63). Altogether, these data suggest that besides the hippocampal impairments, VAD could also induce amygdala dysfonctionning resulting in fear memory deficits. Thus, it could be interesting to test the effects of VAD in an amygdala-dependent task with a cued fear conditioning paradigm to clarify this point.

Brain glucocorticoid levels can be influenced (i) by the activity of the HPA axis but also (ii) by the activity of 11β-HSD1 amplifying intracellular steroid action (64). Thus, other studies have suggested a central role of 11β-HSD1-generated glucocorticoids in hippocampus-dependent memory processes (18). Indeed, it has been shown that 11β-HSD1 levels increase with age in hippocampus correlating with impaired cognitive performance in spatial memory tasks (65). As observed in aged rodents, we have previously shown that elevated hippocampal CORT levels evaluated in basal conditions probably resulting from elevated expression and hyperactivity of hippocampal 11β HSD1 could contribute to spatial memory deficits in VAD rats (15). Consistent with this previous work, we show an elevated expression of hippocampal 11β-HSD1 which is completely abolished by vitamin A supplementation. In order to better understand some potential mechanisms involved in the modulation of hippocampal GC activity during VAD, we have studied the expression of some RAR receptors, that have been shown to negatively regulate 11β-HSD1 expression in vitro (66). We focused on RARα and RARβ receptors because (i) RARα, is abundantly expressed in the hippocampus and plays a critical role in synaptic plasticity and hippocampus-dependent memory (67-68), (ii) RARβ is an indicator of RA signaling disturbances and seems down-regulated during VAD in the brain (69) and (iii) we have previously shown that RA treatment induces only a modulation of these two receptors in aged mice, a finding which has been correlated with the amplitude of intrahippocampal CORT levels after novelty-induced stress (21). Thus, associated with the elevated expression of hippocampal 11β-HSD1 in VAD rats, we have shown a decrease in RARβ expression, and an increase of RARα expression in the hippocampus. All these effects are normalized by vitamin A supplementation. This differential regulation of RAR receptor expression with VAD has already been found by immunostaining in the male rat brain (5). Moreover, we previously showed that RA treatment increases hippocampal expression of RARβ in middle-aged rats while RARα is decreased

(21). Interestingly, it has been shown that under conditions of low vitamin A, a local increase of RA could be detected in astrocytes to maintain a source of RA in the brain suggesting that some RA receptors could be up-regulated in VAD conditions (70). The positive correlation evidenced between hippocampal RARα and 11β-HSD1 expression in VAD and VAD supplemented groups suggested that the decrease expression of RARα evidenced in VAD supplemented rats could contribute to normalize 11β-HSD1expression in order to limit CORT levels in the hippocampus. According to these results a decreased expression of RARα has been associated with lower hippocampal CORT release in stressed middle-aged mice (21). Thus, our present results further substantiate our hypothesis of a close relationship between hippocampal retinoid and glucocorticoid pathways and that hippocampal 11β-HSD1 could be under the control of some retinoid receptors. However, other regulation mechanisms are probably involved in the control of 11β-HSD1 expression and activity in VAD rats. Given that 11β-HSD1 expression has been evaluated post-stress and that stress has previously been shown to increase hippocampal 11β-HSD1 expression (71), we can also suggest a direct modulation of the enzyme expression by glucocorticoid receptors. More experiments will be required to clarify the modulation of 11β-HSD1 expression by retinoid and glucocorticoid receptors.

Lifelong deficiency of 11β-HSD1 in mice with elevated plasma CORT levels prevents age-dependent spatial memory impairments (72-73). We previously found that elevated expression of hippocampal 11β-HSD1 associates with fear memory deficits in VAD rats and hypothesized that the inhibition of the enzyme may have a beneficial effect on contextual fear memory in VAD rats. However, the data described herein show that the elevated expression of hippocampal 11β-HSD1 evidenced in VAD rats does not correlate in an increase in hippocampal 11β-HSD1 activity under stressful conditions (open field-induced stress). We suggest that post-translational regulatory mechanisms of the enzymatic activity could be involved in this effect in order to limit local CORT production after stress and to compensate for VAD-induced basal 11β-HSD1 hyperactivity observed in a previous study (15). In contrast to results observed with vitamin A supplementation, a single injection of UE2316 at the time of training cannot prevent fear conditioning performance deficits in VAD rats and, instead, impairs memory for contextual fear conditioning regardless of the vitamin A status. Moreover, UE2316 treatment-induced fear memory impairments have been associated with reduced plasma CORT levels following stress regardless of the vitamin A status with a slight decrease in hippocampal 11β-HSD1activity in VAD rats. According to our

results, the inhibition of 11β-HSD1 by the same treatment has recently been shown to impair contextual fear memory in control mice but also to reduce plasma CORT levels after stress (20, 23). We have suggested previously that blunted plasma CORT release during fear conditioning training could be involved, at least in part, in VAD-induced fear performance deficits leading to altered fear memory processing. As UE2316 treatment does not interfere with training performances of control and VAD rats but reduces plasma CORT levels after stress, interference with the acute rise of GC induced by fear training during consolidation processes could be also involved in UE2316-induced fear memory deficits. Indeed, since post-training administration of CORT enhances fear memory consolidation via activation of GRs located in the hippocampus (74), lowered intrahippocampal CORT levels associated with 11β-HSD1 inhibition during consolidation processes (75), may in part underlie the impaired contextual fear memory in control and VAD rats. This hypothesis is also substantiated by our data showing reduced plasma CORT levels measured after fear conditioning training in UE2316-treated control rats and a decreased hippocampal 11β-HSD1 activity more particularly in UE2316-treated VAD rats. Furthermore as consolidation of fear memory is also enhanced by GR activation in the amygdala (53, 76), we cannot exclude an inhibitor treatment effect on CORT levels in this brain region. Interestingly, although contextual fear conditioning is significantly reduced by a short-term UE2316 treatment in aged mice, the same treatment improved spatial memory in these cognitively impaired aged rodents (20). Thus, as inhibition of 11β-HSD1 differentially modulates spatial and fear memories in aged rodents, known to exhibit a natural decrease in RA signaling pathway (13), it will be very interesting to test the preventive effect of UE2316 treatment in our VAD rats on a spatial memory task.

In summary, these data demonstrate for the first time that VAD alters contextual fear conditioning, another memory task requiring the integrity of the hippocampus. This deficit consisted of an altered acquisition of the conditioned response with a poor contextual fear memory. These VAD-induced cognitive impairments have been associated with elevated basal plasma CORT levels, a saturated CORT release following stress and altered hippocampal retinoid receptors and 11β-HSD1 expression. Finally, even if vitamin A supplementation can normalize the fear conditioning performances and all biochemical parameters, this supplementation cannot correct contextual fear memory deficits. Since glucocorticoids are necessary for memory processing of aversive events, a dysregulation of HPA axis during training but also during consolidation processes is probably involved in VAD-induced contextual fear memory impairments. Indeed, an acute inhibition of 11β-HSD1

by a single injection of UE2316 which cannot restore fear conditioning performances in VAD rats impairs contextual fear memory and reduces plasma CORT levels following stress regardless of the vitamin A status. Future studies are required to better determine the dietary forms and/or the doses of vitamin A that could be recommended to prevent fear memory deficits and to clarify whether the modulation of glucocorticoid pathway by vitamin A status could be one of the biological mechanisms by which retinoids can exert their effects on hippocampal plasticity and function.

Acknowledgements

The authors thank Dominique Panzeri, Nathalie Argenta, for animal care, Nathalie Arvy for 11β-HSD1 analyses. The authors have no conflicts of interest to declare. This work was supported by the Region Aquitaine, CNRS and the French National Institute Of Food Processing (INRA).

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Figure legends

Figure 1: Experimental design. In the experiment 1, we have studied the effects of vitamin A status (deficiency and supplementation) on contextual fear conditioning, locomotor activity, total plasma CORT levels and hippocampal retinoic acid receptors and 11β-HSD1 mRNA expression. In the experiment 2, we have tested the effects of a single intraperitoneal injection of UE2316 (UE2316, 10 mg/kg), the selective inhibitor of 11β-HSD1, on contextual fear conditioning, locomotor activity, total plasma CORT levels and hippocampal 11β-HSD1 activity. All groups were euthanised 20 min after the open field test and blood samples and hippocampi were collected for further post-stress biochemical and molecular analyses.

Figure 2: Effects of 10-week VAD on spontaneous locomotor activity in the actimetry test. 10 weeks after their arrival, the spontaneous locomotor activity of the rats was recorded for 30 min in actimetry cages. Blocks of 5 min were considered for statistical analysis. The actimetry index indicated that a 10-week VAD does not induce alterations in global locomotor activity.

Figure 3: Effects of VAD and vitamin A supplementation on contextual fear conditioning. At 12-week VAD, animals were trained in a fear conditioning chamber. **(A) Training.** Freezing data over the eight min of the fear conditioning. **(B) Contextual fear memory.** Twenty-four hours after conditioning, contextual fear memory was assessed by placing rats again in the same conditioning chamber and freezing data over the first 5 min in the conditioning chamber were recorded. **(C)** Twenty-four hours after the context test, each animal was tested in another chamber than the one used for conditioning. A tone test was conducted to examine whether rats would express fear to the stimulus. VAD rats exhibit impairments during the conditioning and the context test while the freezing response during the tone test is normal. Vitamin A supplementation reverses the freezing level during the training but cannot suppress contextual fear memory deficits in VAD rats. * $p<0.05$ VAD vs. Control, *$p<0.05$ deficiency effect by ANOVA. $n = 8–10$ per group.

Figure 4: Effects of VAD and vitamin A supplementation on (A) locomotor activity in the open field test, (B) total plasma CORT levels and (C) adrenal glands index. (A) The

path length travelled by rats was recorded for 10 min in the open field test. 20 min after the end of the open field test, all groups were euthanised and blood samples were collected to measure post-stress total plasma CORT levels and the index of adrenal glands in the different groups. (B) Total plasma CORT levels in basal and stress conditions. (C) The index of adrenal glands was expressed as the ratio of adrenal glands weight to 100g of body weight. VAD induces an increased basal plasma CORT levels, a blunted CORT release following stress and an hypertrophy of adrenal glands. All these effects are normalized by vitamin A supplementation. **p<0.01 VAD vs. Control ; °°°p<0.001 VAD vs. Control + VitA; ### p<0.001 VAD vs. VAD + VitA; ## p<0.01 VAD vs. VAD + VitA; ¤¤¤ p<0.001 basal vs post-stress in each group. ANOVA followed by Fischer's post-hoc tests. n= 8–10 per group.

Figure 5: Effects of VAD and vitamin A supplementation on hippocampal RARα, RARβ and 11β-HSD1 mRNA expression. 20 min after the end of the open field test, all groups were euthanised and hippocampi were collected to measure (A) RARα, (B) RARβ and (C) 11β-HSD1 mRNA expression after stress. VAD decreases hippocampal RARβ mRNA expression but increases hippocampal RARα and 11β-HSD1 mRNA expression. All these effects are normalized by vitamin A supplementation. (D) Correlation analyses between hippocampal expression of RARα and 11β-HSD1expression in VAD and VAD supplemented groups. 11β-HSD1 mRNA expression positively correlates with RARα hippocampal mRNA expression (r = 0.461, p= 0.0356). *p< 0.05 VAD vs. Control; °p < 0.05 VAD vs. Control + Vit A; °°°p < 0.001 VAD vs. Control + Vit A; # p < 0.05 VAD vs. VAD + Vit A; ## p < 0.01 VAD vs. VAD + Vit A; ### p < 0.001 VAD vs. VAD + Vit A. ANOVA followed by Fischer's *post-hoc* tests. n= 8–10 per group.

Figure 6: Effects of a single injection of UE2316 on contextual fear conditioning memory. At 12-week VAD animals were injected intraperitoneally one hour before the training in the conditioning chamber. **(A) Training.** Freezing data over the eight min of the fear conditioning. **(B) Contextual fear memory.** Twenty-four hours after conditioning, contextual fear memory was assessed by placing rats again in the same conditioning chamber and freezing data over the 5 min in the conditioning chamber were recorded. **(C) Tone test.** Twenty-four hours after the context test, a tone test was conducted. Pharmacologic inhibition of 11β-HSD1 reduces contextual fear conditioning memory in all rats. * p < 0.05 deficiency effect, # p<0.05 UE2316 effect by ANOVA. n = 8–10 per group.

Figure 7: Effects of UE2316 on (A) locomotor activity in the open field and (B) total plasma CORT levels and (C) hippocampal 11β-HSD1 activity in relation to vitamin A status. **(A)** UE2316 is injected one hour before the open field test and path length of the rats was recorded for 10 min. 20 min after the end of the open field test, all groups were euthanised and blood samples and hippocampi were collected to measure **(B)** total plasma CORT levels and **(C)** hippocampal 11β-HSD1 activity following stress. Pharmacologic inhibition of 11β-HSD1 reduces plasma CORT levels regardless of vitamin A status and induces a slight decrease in hippocampal 11β-HSD1 activity among VAD rats. * $p < 0.05$ deficiency effect, # $p<0.05$ UE2316 effect by ANOVA. n = 8–10 per group.